The effect of dietary copper exposure to benthic ostracod *Heterocypris incongruens* using whole sediment toxicity test

Utilisation du test de toxicité des sédiments pour l'estimation de l'effet de l'exposition au cuivre alimentaire sur les ostracodes benthiques *Heterocypris incongruens*

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RÉSUMÉ

Cette étude porte sur la bioaccumulation du Cuivre (Cu) par les micro algues vertes *Scenedesmus acutus* et *Chlorella vulgaris* ainsi que sur la toxicité du cuivre alimentaire depuis les algues vers les ostracodes benthique *Heterocypris incongruens. S.acutus* et *C.vulgaris* ont été exposés pendant 10 jours à différentes concentrations de Cuivre présent dans l'eau. Puis les ostracodes ont été nourris avec ces algues pendant 6 jours en l'absence totale de lumière, modifiant partiellement la méthode de test ISO 14371. À mesure que la concentration de cuivre dissout augmente, le Cuivre total présent dans les cellules des micro algues vertes augmente de 12 à 1.1 x 10^4 µgCu/g dw pour *S.acutus et de* 19 à 1.3 x 10^4 µgCu/g dw pour *C.vulgaris*. La majeure partie du Cuivre accumulé était localisée à l'intérieur des cellules. Ces algues exposées au Cuivre ont augmenté la mortalité des ostracodes. La concentration de cuivre dissout dans l'eau extraite à la fin du test était trop basse pour causer la mortalité des ostracodes, ainsi le cuivre alimentaire semble en être la cause la plus probable. Il n'y avait presqu'aucune différence parmi les différentes fractions (insoluble, soluble et échangeable) permettant de déterminer le possible effet toxique du cuivre sur les ostracodes. De ce fait, le cuivre total aussi bien que le cuivre trophique disponible (soluble et échangeable) peuvent être utilisés pour montrer l'effet du cuivre alimentaire sur les ostracodes. La relation dose - réponse entre le cuivre alimentaire et la mortalité des ostracodes pourrait être utile pour l'interprétation des test de toxicité sur les ostracodes dans de nombreuses applications. À ce jour, l'effet du cuivre alimentaire sur les ostracodes *H.incongruens* n'avait pas été rapporté.

ABSTRACT

In this study, we investigated the bioaccumulation of copper (Cu) by microgreen algae *Scenedesmus acutus* and *Chlorella vulgaris* and toxicity of dietborne copper from algae to benthic ostracod *Heterocypris incongruens*. *Scenedesmus acutus* and *C. vulgaris* were exposed to different concentrations of waterborne Cu for 10 days then the ostracod was fed this food exclusively under 24-h dark condition for 6 days, partially modifying the test method recently published as ISO 14371. Total copper in the cells increased with increasing dissolved copper concentrations from 4.8 to 4.5 x 10^3 µgCu/g dw and 10 to 5.3 x 10^3 µgCu/g dw for *S. acutus* and *C. vulgaris*, respectively. Most of the accumulated Cu were located inside the cells. The Cu-exposed algae increased the mortality of the ostracod. The concentration of dissolved copper in the overlying water at the end of the test was too low to cause the mortality of ostracod, thus the dietborne Cu was most likely the cause. There was almost no difference among the different fractions to determine the possible toxic effect of dietary Cu to ostracod thus, total or trophically available copper can be used to show the dietary Cu effect to ostracod. The dose-response relationship between the dietborne Cu and ostracod mortality would be useful in interpreting the results of ostracod toxicity tests in various applications. To our knowledge, the effect of dietborne Cu to ostracod *H. incongruens* has not been reported.

KEYWORDS

Algae, Copper, Dietary exposure, Ostracod, Sediment toxicity test

1 INTRODUCTION

Stormwater runoff from urbanized environment can cause several adverse effects on water quality in the receiving environment, such as deposition of contaminated sediments, toxicity from contaminants related to traffic, nutrient enrichment and eutrophication, and overall water quality degradation (Bartlett et al., 2012). A number of pollutants are associated with urban stormwater runoff such as nutrients, bacteria, suspended solids, heavy metals, hydrocarbons and pesticides (Hofman et al., 1984; Krein and Schorer, 2000; Furumai et al., 2002; Eriksson et al., 2005; Camponelli et al., 2010; Nakajima et al., 2010) and accumulation of these pollutants in water environment results in toxicity to aquatic organisms. Trace metals are of particular importance due to their abundance in the environment as well as their potential toxicity. Understanding the transport of metals and their distribution in water and sediments is important in determining whether these metals might impact aquatic organisms inhabiting the area.

One of the major challenges in ecotoxicology is how to relate the results of the short term toxicity test to actual long-term effects on the aquatic community. In real aquatic environments, metals can be taken up by aquatic organisms via several potential routes, including absorption across respiratory organs, dermal absorption, sediment ingestion, and food ingestion. For instance, the effects of stormwater runoff on the aquatic environment are particularly difficult to assess due to many widely fluctuating stressors (Burton, 1995). Furthermore, stormwater runoff may present a short-term problem, when pulses of contaminants enter a receiving system, and a long-term problem, when toxicants accumulate in the sediment (Burton and Pitt, 1999). Contaminants in stormwater runoff accumulate in sediments and can be mobilized by storm events. Several approaches were done to study the impact of contaminated sediments and stormwater (Hatch and Burton, 1999; Rosenkranttz et al., 2008; Bartlett et al., 2012). Most of the recent studies on the other hand, have addressed the toxicity of stormwater using ecotoxicological test with single or multiple species in either a single ecotoxicological approach, or included in an integrated approach (Tixier et al., 2011).

A newly standardized ostracod toxicity test (ISO14371) (ISO, 2012) is one of the available bioassays to evaluate the toxicity in the sediment as well as stormwater runoff. It is a simple and low cost solid phase assay for routine monitoring of freshwater sediments. A direct contact sediment toxicity test method was developed by Dr Persoone et al. (Chial and Persoone, 2002a, b) and it was recently standardized by the International Organization for Standardization as ISO14371 (ISO, 2012). This method has been applied to several different solid samples such as sediments (Chial and Persoone, 2002c; Watanabe et al., 2008; Tsakovski et al., 2012), soils (Chial and Persoone, 2003; Santorufo et al., 2012), and urban road dust (Watanabe et al., 2011). Metal toxicity for ostracods via aquatic exposure has been summarized (Kudlak et al., 2011) and aquatic Cu exposure results were used as reference data in the ISO standardization procedure (ISO, 2012). To the best of our knowledge, however, the effect of dietborne Cu on the ostracod *H. incongruens* has not been studied. Similar research has been conducted with different freshwater invertebrate (De Schamphelaere et al., 2007) but we hypothesized that dietary Cu exposure would also be toxic to *H. incongruens*. Systematic dietary exposure toxicity data are expected to be useful for interpreting the results of ostracod sediment toxicity testing. A proper solid phase toxicity assessment is of importance but there is significant lack of information on solid-phase dose response relationship that can relate the results of sediment toxicity test and sediment contaminant content possible due to stormwater runoff.

A proper solid phase toxicity assessment is of importance in stormwater management but there is significant lack of information on solid-phase dose response relationship for the test organisms. The present study investigated the toxicity and trophic transfer of dietary Cu from freshwater microgreen algae, *Scenedesmus acutus* and *Chlorella vulgaris,* to the benthic ostracod *H. incongruens. Scenedesmus acutus* and *C. vulgaris* were exposed to waterborne Cu for ten days to prepare the copper-contaminated food and the ostracod was fed exclusively with this food for six days. The toxic effects of dietborne Cu on ostracod mortality were assessed. In addition, we determined the distribution of Cu incorporated in algal cells and the potential fraction of Cu available for trophic transfer to the ostracod.

2 MATERIALS AND METHODS

2.1 Test organisms

Freshwater microgreen algae, *S. acutus* (NIES-94) and *C. vulgaris* (NIES-2170), were purchased from

the Microbial Culture Collection of the National Institute for Environmental Studies, Japan. *Scenedesmus acutus* and *C. vulgaris* were used in this study as representative food organisms for the ostracod and as primary producers in an urban freshwater environment. The algae were cultured in the laboratory using culture medium (C medium), which was prepared according to the instructions supplied by the culture collection (NIES, 2004). The algal cultures were placed in a light incubator and transferred into fresh media every seven days to maintain the stock in a fresh growth period (exponential growth). The culture conditions were as follows: temperature maintained at 25 ± 2°C, fluorescent light intensity of 5000 lx, and a photoperiod of 12 h light and 12 h dark (12h:12h).

Cysts of *H. incongruens* were obtained from MicroBioTests Inc., Belgium. Dormant *H. incongruens* cysts were hatched in a petri dish with standard freshwater according to the manufacturer's instructions, followed by incubation at 25 ± 1°C with continuous illumination (3000–4000 lx) for 52 h, then pre-feeding with 1.3 mg/mL *Spirulina* (MicroBioTests Inc.) 4 h prior to collecting ostracod neonates.

2.2 Algal incubation

Analytical grade copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) was used as toxicant dissolved in C medium. A series of different Cu concentrations (C0 = 1.2, C1= 8.4×10^2 and C2 = 1.3×10^3 (µg Cu/L) as measured concentration) were prepared. Then, 200 mL of culture media were transferred to 300 mL Erlenmeyer flasks with a sponge plug, which were autoclaved for 15 min at 121°C and allowed to cool to room temperature. Four milliliters of the fresh *S. acutus* and *C. vulgaris* cultures were inoculated into each flask. Each test used two replicate flasks for metal fractionation and chemical analyses. The algae were incubated in a light incubator at 25 ± 2°C for ten days with a light–dark cycle of 16h:8h (light intensity of 2000–3000 lx).

2.3 Cu fractionation in algal cells

Metal in the water could be taken up by algae and distributed throughout several fractions of the algal cells. Metal fractionation of the algal cells was conducted using published method (Amiard-Triquet et al., 2005), with some modifications (Resnawati, 2009; Sevilla, 2012). After ten days incubation, the algae samples were collected and their different metal fractions (i.e. cell-surface exchangeable and intracellular soluble fractions) were measured by ICP-MS.

Two replicate flasks were combined and a known volume (400 mL) of the combined algal suspension was centrifuged at 2000 rpm for 10 min to separate the algal cells (Pellet 1) from the supernatant (SN1). Cu in the SN1 corresponded to the total dissolved Cu in the test system whereas Cu in Pellet 1 was the total Cu in the whole algal body. Pellet 1 was dried in an oven at 85°C for 3 h to measure the total Cu concentration on a dry weight basis. The dried algae were weighed using an electric balance to determine the algal dry weight. These samples were digested in 10 mL nitric acid (60%) at 140°C for 10 min using a microwave digestion system (Anton Paar Multiwave 3000). The time required to reach the target temperature was set at 20 min to digest the samples using a gradual temperature increment. The digested samples were diluted 50 times to reduce the HNO_3 concentration to 1.2% before subsequent Cu determination by ICP-MS.

Another Pellet 1 (collected similarly by centrifugation) was resuspended in a chelating agent (10 mL of 100 µg/L EDTA) and kept for 10 minutes. Franklin et al. (2000) showed that EDTA treatment did not cause cell lysis. The suspended cells were again separated by centrifugation (2000 rpm for 10 min) and this supernatant (SN2) was defined as the cell-surface exchangeable fraction. The settled pellet (Pellet 2) was dispersed in 5 mL of 0.02 M TRIS buffer for 15 min and samples were then sonicated (100 W for 15 min) to disrupt the algal cells. The homogenate was ultracentrifuged (30,000 × *g* for 1 h at 4°C). Cu in the supernatant (SN3) was defined as the intracellular soluble fraction whereas the Cu in the pellet (Pellet 3) was referred to as the intracellular insoluble fraction. Pellet 3 was practically impossible to be recovered for Cu measurement due to its small mass, so the Cu content of Pellet 3 was calculated theoretically based on the difference between Pellet 1 and the sum of SN2 and SN3.

2.4 Algal food preparation

Cu-contaminated algae *S. acutus* and *C. vulgaris* were harvested after ten days exposure to different concentrations of Cu, as described in section 2.2. The absorbance of the algal suspension was measured at 560 nm using a Hitachi U-2010 spectrophotometer, and the initial cell concentrations were computed based on the linear relationship between the absorbance and the cell concentrations. The algae were recovered by centrifugation at 3500 rpm for 15 min and rinsed with standard freshwater (2–3 times). Finally, the pellet was dispersed into the designated volume of standard

freshwater to adjust to the necessary algal concentration for feeding the ostracod. The final algal cell concentrations were adjusted to 1.5×10^7 cells/mL (*S. acutus*) and 3.0×10^8 cells/mL (*C. vulgaris*).

2.5 Toxicity test: dietary exposure of ostracod to Cu

The materials and test procedures followed the standard operational procedure used in the ostracod crustacean *H. incongruens* toxicity test (ISO, 2012), with some modifications. Fine granular quartz sand (Merck Co.), which had been washed and calcinated, was used as the reference sediment. The sand particle size was 0.2–0.8 mm (≥40%). The dietary exposure experiment involved giving the Cu-contaminated algae to the ostracod as food during a six-day toxicity test and incubating at 25 ± 1°C under 24-h dark condition. The bioassays were performed in six cup polystyrene multiwell plates with 10 ostracods per well using six replicates (six wells), containing 1 mL of quartz sand, 2 mL standard freshwater, and 2 mL of the prepared contaminated algal food suspension.

As a reference, ostracods were also exposed (6 days) to different Cu concentrations via an aqueous phase as follows: 2.6×10^2 (AE1), 4.6×10^2 (AE2), 8.1×10^2 (AE3; close to the initial state of C1), 1.4×10^3 (AE4; close to the initial state of C2), and 2.6×10^3 (AE5) (µg Cu/L) as measured concentration. The tests were conducted using uncontaminated *S. acutus* as food under 24-h dark condition.

The ostracod body lengths were measured using a microscope at the beginning and the end of the six-day test. Ostracod mortality was measured after the six-day exposure period. The toxicity test was considered valid when the following validity criteria (ISO, 2012) were met. The percentage mortality in the control sediment was ≤ 20%, and the mean length of the ostracods in the control sediment was ≥1.5 times greater than the mean length of the test organisms at the start of the test.

2.6 Determination of Cu by ICP-MS

All samples for Cu analysis were filtered through 0.45 µm PTFE membrane disposable filter units (Advantec, 25HP045AN). An internal standard (yttrium) was added to all samples before measurements to correct for any possible variations during the analysis. Cu was determined using ICP-MS (Agilent 7500cx), and samples were prepared in diluted acid (60% HNO_3 for metal analysis grade; WAKO) at 1.2% v/v.

2.7 Statistical analysis

The experimental data were analyzed using PASW Statistics version 18. The Cu toxicity was expressed as the median lethal concentration (LC50) using probit analysis.

3 RESULTS AND DISCUSSION

3.1 Cu fractionation of the algal cells

After ten days incubation with C1, 37–38% of the initial Cu was taken up by the two algal species and $5.2–5.3 \times 10^2$ µg Cu/L remained in the liquid phase. An increase in the initial dissolved Cu with C2 increased the total amount of Cu taken up by the algae to almost 51–53% of the initial Cu while the remaining Cu was $6.1–6.3 \times 10^2$ µg Cu/L.

The distribution of Cu in the cell-surface exchangeable, intracellular soluble, and intracellular insoluble fractions of the *S. acutus* and *C. vulgaris* cells is shown in Figure 1. The percentage of cell-surface exchangeable Cu was low in the two algal species, i.e., only 3% at highest Cu concentration. Thus, the Cu was mainly present inside the cells as intracellular soluble and insoluble fractions in both algal species. At C0, the incorporated Cu was mainly in an insoluble form (89%) for *S. acutus* whereas *C. vulgaris* stored Cu almost 94%. With higher initial dissolved Cu concentrations, the Cu incorporated in the algal cells also increased. In the C1 and C2 conditions, *S. acutus* stored 30% of Cu in the intracellular soluble form and 63-67% in the intracellular insoluble form. *Chlorella vulgaris* accumulated 27-58% in its intracellular soluble fraction and 37-70% in its intracellular insoluble fraction. There was a slight difference between the two species in terms of the different Cu fractions accumulated in their algal cells with increasing dissolved Cu. The hypothetical trophically available Cu (i.e. intracellular soluble + cell-surface exchangeable) ranged 30-63% under C1 and C2 conditions. It was noted that, under C1 condition, the hypothetical trophically available Cu content was higher in *C. vulgaris* than in *S. acutus*.

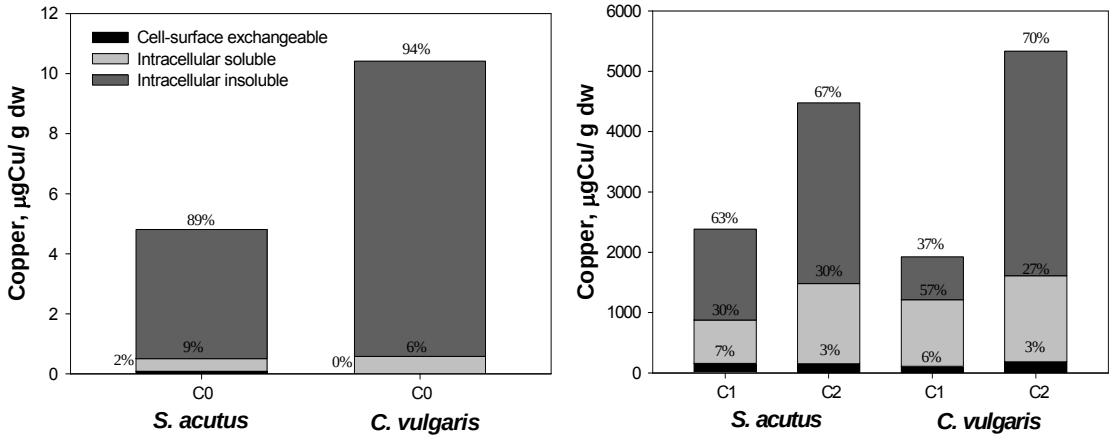

Figure 1. Different copper fractions in the algal cells of *Scenedesmus acutus* and *Chlorella vulgaris*. The copper distributions (%) in the cells are shown in each bar.

3.2 Ostracod exposure to aquatic and dietary Cu

Figure 2 shows the relationship between aqueous Cu concentration and ostracod mortality. As shown in the previous section, the food algae could grow even under C2 condition (close to AE4 which caused more than 50% mortality to ostracod) and the Cu concentration after the algal growth decreased to between AE2 (4.6×10^2 µg Cu/L) and AE3 (8.1×10^2 µg Cu/L) conditions which was not toxic in ostracod mortality. The ostracod species was more sensitive to dissolved copper than the algal species.

The LC50 of the dissolved Cu was calculated as 1.2×10^3 µg Cu/L. This was comparable to the interlaboratory trial result (6.58 mg/L as $CuSO_4 \cdot 5H_2O$, i.e., 1.67 mg Cu/L) given in Annex of the ISO protocol (ISO, 2012) and also with the published value of 0.014 mmol/dm^3 (0.89 mg Cu/L) (Kudlak et al., 2011). This slight difference might be because of the differences in the ionic strength or cationic concentrations in the test water, i.e., Kudlak et al. (2011) used distilled water instead of standard freshwater, whereas the ISO interlaboratory trial used the reference sediment provided by MicroBioTests Inc. instead of quartz sand. The reference sediment appeared to release calcium during the six-day test according to our preliminary observations (data not shown).

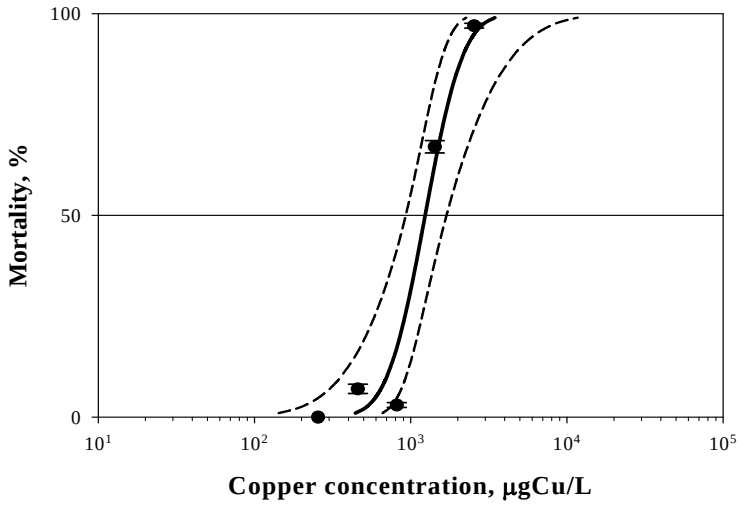

Figure 2. Dose response relationship of aqueous copper exposure in ostracod mortality under 24-h dark condition, The dashed lines indicate the 95% confidence upper and lower limits of the regression. Error bars represent standard deviation.

Figure 3 shows the dose–response relationships between the total Cu contents of *S. acutus* and *C. vulgaris* cells and the percentage ostracod mortality after six days dietary exposure. The results showed that contaminated food combined with clean sediment affected the ostracod mortality. The ostracod mortalities with clean algae (C0; 4.8 µgCu/g dw for *S. acutus* and 10.4 µgCu/g dw for *C. vulgaris*) were 12% and 3%, respectively, which met the criteria specified for the control conditions (ISO, 2012). In the C1 and C2 tests, a significantly elevated mortalities were observed for ostracod fed with Cu-exposed algae. The Cu concentrations measured in the overlying water after the six-day toxicity tests using Cu-exposed *S. acutus* and *C. vulgaris* are shown in Table 1. The Cu concentration in the aqueous phase was increased by the addition of contaminated food, so Cu in the food particles may have been released into the test system to some extent. It was also possible that ostracods released Cu into the test system via excretion during the exposure period. However, the Cu concentrations in Table 1 were too low to cause ostracod mortality, as shown in Figure 2. Therefore the observed ostracod mortality in the tests with contaminated algae should be considered to be a result of Cu in the food, rather than Cu released into the water.

Table 1. Copper concentration in the overlying water after the 6-day dietary exposure using Cu-contaminated *Scenedesmus acutus* and *Chlorella vulgaris* as food.

Test	Cu concentration in overlying water after the test, µgCu/L	
	Cu-exposed ***S. acutus***	Cu-exposed ***C. vulgaris***
C0	4.0	3.9
C1	32	26
C2	32	37

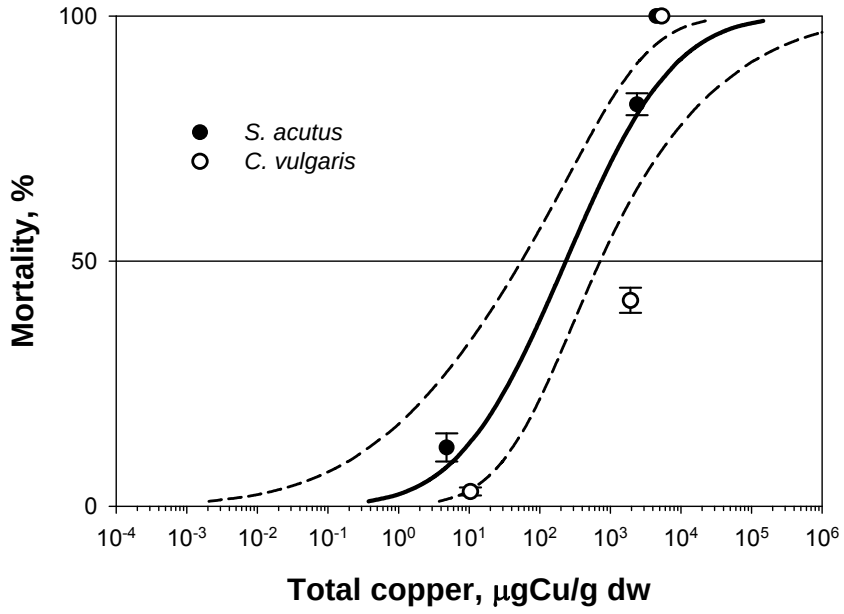

Figure 3. Dose response relationship of total copper in *Scenedesmus acutus* and *Chlorella vulgaris* cells and percent mortality of ostracod after the 6-day dietary exposure. The regression was conducted for all the six plots regardless of the species difference. The dashed lines indicate the 95% confidence upper and lower limits of the regression. Error bars represent standard deviation.

As shown in the previous section, the Cu concentration under C1 condition of contaminated-food preparation decreased from 8.4×10^2 to $5.2–5.3 \times 10^2$ µg Cu/L. Both the initial and last concentrations of copper would not cause ostracod mortality (Figure 2), but the algal cells were toxic through the dietary exposure (Figure 3). Under C2 condition, the contaminated-food caused 100% mortality (Figure 3) although the initial concentration for food preparation (1.3×10^3 g Cu/L) might cause just a measurable mortality and the final concentration ($6.0–6.3 \times 10^2$ µg Cu/L) was below toxic level (Figure 2). The result suggested that Cu toxicity under such extremely polluted conditions might be caused primarily by dietary exposure rather than waterborne exposure although the initial Cu concentrations to which the algae were exposed ($\geq 8.4 \times 10^2$ µg Cu/L) are found only rarely in natural environments.

The current study used only a simple system with a single-consumer (ostracod) grazing on a single algal food (*S. acutus* or *C. vulgaris*). However, the accumulation of metals by algae (primary producers) in aquatic environments may be species-specific. Thus, the dietary Cu dose at a given waterborne concentration in a natural system may be highly dependent on the local algal species community as well as on the food preference of local crustaceans (De Schamphelaere et al., 2007). Thus, it is recommended that the inter-species variability in their sensitivity to dietary Cu should be determined, as along with the metal accumulation by different algal species and the importance of algal species as food sources.

The speciation of metals in sediments is a critical factor in assessing the potential environmental impacts (Peng, et al., 2004). Aquatic benthic organisms such as ostracod are most likely to come into contact with surface sediments and ingest sediment as well thus the metal burden at the surface is most relevant for the prediction of possible adverse effects. We assumed that only a limited fraction in the food could be assimilated by the ostracod and the fractionation result would provide us a better index than total metal content. The two algal species used in this study (maybe accidentally) gave a relatively similar Cu distribution (Figure 1). Therefore we could not validate the concept of trophically available metal (intracellular soluble + cell-surface exchangeable) in the food as a predictive index of toxicity in consumer species. The metal fractionation method in the algal cells is expected to be utilized to characterize metal fraction and chemical availability of metal in sediments and road dust affected by stormwater runoff since the conventional fractionation (sequential extraction) method is tedious and time-consuming. Further data accumulation with different algal species and under various food preparation conditions is required for proposing the metal fractionation method in the current study as an inexpensive and rapid assessment of bioavailability of metals in particulate pollutants.

4 CONCLUSION

Scenedesmus acutus and *C. vulgaris* were exposed to different levels of Cu concentrations to determine the different fractions of metal in algal cells and the amount of Cu that was trophically available for possible transfer to a benthic ostracod. Both algae accumulated most of the Cu in their algal cells in intracellular soluble and insoluble forms. The proportions of hypothetical trophically available Cu in their cells were 6 to 63%. The dietary exposure of *H. incongruens* to the Cu-contaminated algae resulted in increased mortality. It was demonstrated that the toxic effects were due to the dietborne Cu in the algal food, not due to the indirect (released) waterborne Cu exposure. The comparison between the aquatic and dietary exposure test results shows that the ostracod was more sensitive to Cu-contaminated algal food than dissolved Cu. The dose–response relationship of solid phase Cu exposure may be useful to users of the ostracod toxicity test.

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